EXHIBIT 3

Diagnostic Molecular Microbiology

PRINCIPLES AND APPLICATIONS

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extension reactions terminate at various distances from the primer, resulting in amplification products that contain opposite DNA strands of indeterminate lengths. However, after the second cycle, a specific amplification product begins to accumulate and rapidly becomes the predominant product. This so-called short product of PCR comprises the two primer-binding sites along with the stretch of intervening nucleic acids. It is identified by its precisely defined length and the presence of amplified internal target sequences. Nonspecific amplification products (that is, spuriously amplified sequences that do not contain the specific target sequence) are only rarely the same size as the target-specific product and do not contain internal sequences that are homologous to target-specific hybridization probes.

In conventional hybridization with a single oligonucleotide probe, imperfect pairing of bases allows the probe to anneal in a nonspecific manner so that it binds to multiple nontarget sites in the DNA mixture. Indeed, for low-abundance targets (especially if the hybridization is done at low stringency), the contribution of background hybridization may exceed that of the true target sequence. PCR overcomes much of this problem in part by requiring two specific hybridization events such that the two oligonucleotide primers each initiate DNA synthesis toward the annealing site for the primer on the opposite strand. Furthermore, to provide efficient amplification of the target sequence, the two priming sites must be within reasonable proximity of one another. Thus, the physical linkage of two priming sites by DNA synthesis (which itself places great demands on the stringency of primer-target hybridization) results in the primer-directed synthesis of new priming sites. This critical element of design gives PCR much of its sensitivity and specificity. In fact, the contribution of multiple independent specific hybridization events to the amplification process is the basis for the specificity of most of the amplification methods described in this section.

Many technical improvements have been made so that the PCR of today only remotely resembles, on an operational basis, its earlier counterpart. These improvements also enhanced greatly the analytical sensitivity and specificity of the technique. The first was the use of a thermostable DNA polymerase (*Taq* polymerase) isolated from a thermophilic bacterium, *Thermus aquaticus*, that grows in hot springs at temperatures of 70 to 75°C (135). The enzymatic activity of *Taq* polymerase has a half-life of approximately 40 min at 95°C and thus is able to withstand the repeated thermal cycling of PCR. Earlier versions of PCR required the addition of fresh polymerase to replenish the enzyme that had been denatured during the heating step. *Taq* polymerase is added once at the beginning of the reaction, thus greatly simplifying sample handling. In addition, conducting the annealing and extension reactions at higher temperatures significantly decreases nonspecific amplification. The annealing step, which is now performed at elevated temperatures, can be customized for each primer set so that conditions are unfavorable for the formation of imperfect base pair hybrids of primer and target (135).

A second innovation was the development of the programmable thermal cycler. The thermal cycler, essentially a programmable heating block, is capable of conducting successive heating and cooling cycles unattended, and it eliminates the tedious task of transferring reaction tubes between water baths or heating blocks set at the requisite temperatures (one of the least envied tasks of graduate students in the 1980s).

PCR is an extremely powerful technique for finding the nucleic acid equivalent of the proverbial needle in the haystack. PCR can be used to selectively